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Flavin Adenine Dinucleotide Binding is the Crucial Step in Alcohol Oxidase Assembly in the Yeast *Hansenula polymorpha*

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We have studied the role of flavin adenine dinucleotide (FAD) in the *in vivo* assembly of peroxisomal alcohol oxidase (AO) in the yeast *Hansenula polymorpha*. In previous studies, using a riboflavin (Rf) autotrophic mutant, an unequivocal judgement could not be made, since Rf-limitation led to a partial block of AO import in this mutant. This resulted in the accumulation of AO precursors in the cytosol where they remained separated from the putative peroxisomal AO assembly factors. In order to circumvent the peroxisomal membrane barrier, we have now studied AO assembly in a peroxisome-deficient/Rf-autotrophic double mutant ($\Delta per1.rif1$) of *H. polymorpha*. By sucrose density centrifugation and native gel electrophoresis, three conformations of AO were detected in crude extracts of $\Delta per1.rif1$ cells grown under Rf-limitation, namely active octameric AO and two inactive, monomeric forms. One of the latter forms lacked FAD; this form was barely detectable in extracts wild-type and $\Delta per1$ cells, but had accumulated in the cytosol of *rif1* cells. The second form of monomeric AO contained FAD; this form was also present in $\Delta per1$ cells but absent/very low in wild-type and *rif1* cells. *In vivo* only these FAD-containing monomers associate into the active, octameric protein. We conclude that in *H. polymorpha* FAD binding to the AO monomer is mediated by a yet unknown peroxisomal factor and represents the crucial and essential step to enable AO oligomerization; the actual octamerization and the eventual crystallization in peroxisomes most probably occurs spontaneously.

KEY WORDS — alcohol oxidase; flavin adenine dinucleotide; peroxisomes; *Hansenula polymorpha*; protein translocation; protein assembly

INTRODUCTION

Microbodies (peroxisomes) play an essential role in methanol metabolism in yeasts, because they contain the key enzymes (alcohol oxidase (AO) and dihydroxyacetone synthase) of both the methanol dissimilatory and assimilatory pathways (Veenhuis *et al.*, 1976; Douma *et al.*, 1985). Alcohol oxidase is an octameric protein of 600 kDa, consisting of eight identical subunits, each of which contains one flavin adenine dinucleotide (FAD) binding site (Kato *et al.*, 1976). Precursors of AO are synthesized in the cytosol and post-translationally imported into the target organelle where assembly and activation is supposed to take place (Bellion and Goodman, 1987;

van der Klei *et al.*, 1991a). Previously, using a riboflavin (Rf) autotrophic mutant of *Hansenula polymorpha* (*rif1*), we showed that AO activation inside peroxisomes was dependent on the availability of FAD (Evers *et al.*, 1994). However, whether the previous binding of FAD to AO monomers indeed was a prerequisite to enable AO oligomerization/activation could not be decided since FAD-limitation not only caused a defect in AO oligomerization but also led to a partial block of AO import into peroxisomes. Hence, in the *rif1* mutant a portion of the monomeric AO was in a different compartment (the cytosol) from the putative assembly factors, which resided inside peroxisomes (Evers *et al.*, 1994). Hence, the only way to analyse properly the role of FAD in the *in vivo* AO assembly pathway was to circumvent

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the peroxisomal membrane barrier. In order to achieve this, we took advantage of the fact that many peroxisome-deficient (*per*) mutants of *H. polymorpha* are available (Cregg *et al.*, 1990). In *per* mutants AO is normally synthesized and active, indicating that the putative assembly factors also function efficiently in the cytosol (van der Klei *et al.*, 1991b). Therefore, we constructed a *per.rif1* double mutant and studied in depth the AO assembly in this mutant at Rf-limiting conditions. The results of these studies are presented in this paper.

MATERIALS AND METHODS

Organisms and growth conditions

The following *H. polymorpha* strains were used: the NCYC 495 (*leu*⁻) wild-type strain, a *per1* deletion strain (Δ *per1* *ura*⁻); Waterham *et al.*, 1994), a *rif1* (*leu*⁻) (Evers *et al.*, 1994) and the double mutant Δ *per1.rif1*. This double mutant was obtained by crossing the Δ *per1* and *rif1* mutants and selecting amongst the progeny for mutants containing both the *rif1* mutation and the *per1* deletion. Cells were grown in batch cultures at 37°C in YPD or mineral medium containing 0.5% (w/v) glucose or 0.5% (v/v) methanol as carbon sources (van Dijken *et al.*, 1976). Prior to the shift to methanol-containing media, cells were extensively pre-cultured on YPD in the presence of 0.6 mM-Rf. Wild-type *H. polymorpha* was grown for 16 h in methanol-containing media, supplemented with 0.2 mM-Rf, *rif1* cells were grown for 24 h. The Δ *per1* and Δ *per1.rif1* mutants were incubated for 48 h in methanol-containing media, supplemented with 0.2 mM-Rf. Growth was monitored by measuring the optical density at 663 nm in a Vitatron colorimeter.

Electron microscopy and immunocytochemistry

Protoplasts were prepared by treatment of whole cells with Zymolyase 20-T (ICN Biomedicals Inc., Costa Mesa, California, U.S.A.; Douma *et al.*, 1985). Immunocytochemistry was performed on ultrathin sections of Unicryl-embedded cells using polyclonal antibodies raised against alcohol oxidase and goat anti-rabbit antibodies conjugated to gold (Amersham, U.K.), according to the method of Slot and Geuze (1984).

Biochemical methods

Crude extracts were prepared as described (van der Klei *et al.*, 1991a). Protein concentrations were

determined according to Bradford (1976). Alcohol oxidase activity was measured according to Verduyn *et al.* (1984).

Octameric AO protein was separated from monomeric protein by sucrose density centrifugation (Bellion and Goodman, 1987). Sucrose gradients were harvested by taking 0.5 ml samples from the top. To determine the amount of monomeric AO as a percentage of the total amount of AO protein, equal volumes of all fractions were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Laemmli, 1970) followed by Western blotting (Kyhse-Andersen, 1984); the blots were decorated with specific antibodies against AO. AO protein levels were determined in all fractions by laser-densitometric scanning of the blots. The percentage of monomeric AO was calculated from the amount of AO protein present in the fractions containing monomeric AO protein (fractions 1–3) and the total amount of AO protein present in all fractions (1–7). Non-denaturing gel electrophoresis using 4–10% linear gradient gels was performed according to Musgrove *et al.* (1987). Native AO was dissociated into monomers by incubation in 80% (v/v) glycerol for 10 min at room temperature (Evers *et al.*, 1995). FAD was removed from octameric AO by incubation of whole cells or crude extracts with 6 mM-KCN for 2 h at 37°C (van der Klei *et al.*, 1989b). The presence of FAD was visualized by illumination of the non-denaturing gels with UV light (Bystrykh *et al.*, 1989). ATP levels in whole cells were determined by ³¹P NMR (Nicolay *et al.*, 1987).

RESULTS

Alcohol oxidase assembly

As described previously, Rf-limitation resulted in reduced levels of AO protein in methanol-induced cells of the *H. polymorpha rif1* mutant (Evers *et al.*, 1994). Similar results were now obtained with the Δ *per1.rif1* double mutant; however, in Δ *per1* cells the AO protein levels were similar to those of wild-type controls (Figure 1). Also, the specific AO activities in crude extracts of both strains containing the *rif* mutation were strongly reduced (Figure 1), indicating that in both strains AO activation was partially prohibited.

Sucrose density centrifugation revealed the presence of monomeric AO protein in crude extracts of methanol-induced cells of all three mutant strains

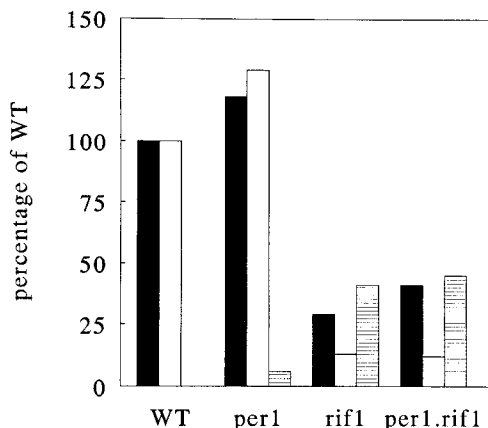


Figure 1. Relative amount of alcohol oxidase (AO) protein (■), specific AO activities (□) and percentages of monomeric AO (▨) in crude extracts prepared from wild-type (WT) and mutant cells of *H. polymorpha*, incubated in methanol-containing media supplemented with 0.2 mM-Rf. The values of wild type are set to 100%. *per1*, *PER1*-deletion strain; *rif1*, riboflavin auxotrophic mutant; *per1.rif1*, double mutant.

(Δ *per1*; *rif1* and Δ *per1.rif1*). In wild-type control cells, monomeric AO was hardly detectable (Figure 2). In Δ *per1* cells, the fraction of monomeric AO was low and amounted to approximately 4% of the total AO protein, but in *rif1* and Δ *per1.rif1*, up to 40% of the total AO protein was present in the monomeric form (Figures 1 and 2). The rates of AO assembly were therefore largely identical in both *rif1* and Δ *per1.rif1* cells.

In methanol-induced cells of Δ *per1* and of the double mutant Δ *per1.rif1*, active AO octamers and inactive AO monomers accumulated in the same compartment, namely the cytosol. Since peroxisomes are absent in these cells, the peroxisomal factors involved in the assembly of AO also reside in the cytosol. In Δ *per1* the rate of AO assembly is only slightly affected, which suggests that the cytosolic location of the putative peroxisomal assembly factor has only a minor effect on the efficiency of peroxisomal protein assembly. Therefore, the accumulation of monomeric AO in Δ *per1.rif1* most probably is largely due to the limitation of its co-factor, FAD. This was also indicated by the fact that in Δ *per1.rif1* the assembly of catalase, a peroxisomal haem-containing protein, was not affected. In all three mutants (Δ *per1*, Δ *per1.rif1* and *rif1*), catalase was invariably present in the assembled, haem-containing tetrameric form (Figure 2). Hence, we conclude that the limiting amount of FAD

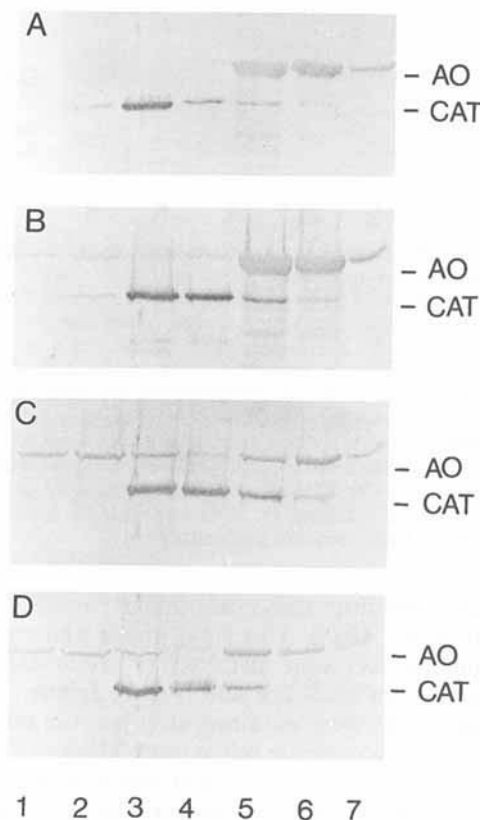


Figure 2. Western blots, decorated with α -AO and α -catalase (CAT) antibodies, prepared from the fractions obtained after sucrose gradient centrifugation of equal amounts of crude extracts of wild-type *H. polymorpha* (A) and mutants Δ *per1* (B), *rif1* (C) and Δ *per1.rif1* (D), showing the presence of distinct amounts of monomeric AO protein in the mutant cells. However, catalase is only present as tetramers. Lane 1 represents the top fraction, lane 7 the bottom fraction of the gradient. Lanes 1-3: monomeric AO (75 kDa); lanes 5-7: octameric AO (600 kDa); lanes 3-5: tetrameric CAT (240 kDa).

in strains containing *rif* mutations affects AO assembly.

^{31}P NMR studies on intact cells showed that the ATP levels were not significantly reduced, compared to wild-type cells, in the mutants grown under FAD-limitation (data not shown). Therefore, it is very unlikely that the accumulation of monomeric AO is due to lowered energy levels.

Monomeric AO is present in two different conformations in Δ *per1.rif1*

To characterize further the conformational states of AO protein present in both mutants containing the *rif* mutation, we performed non-denaturing gel electrophoresis followed by

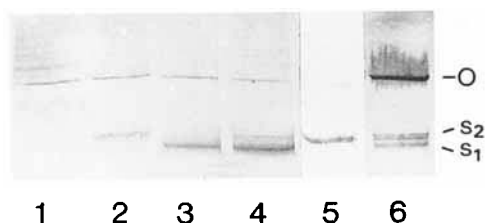


Figure 3. Western blots, decorated using α -AO antibodies, after non-denaturing gel electrophoresis of crude extracts showing the presence of different forms of AO protein. Lane 1: wild-type *H. polymorpha*; lane 2: mutant Δ *per1*; lane 3: *rif1* and lane 4: Δ *per1.rif1*. FAD-containing subunits akin to those present in *per* mutants (lanes 2 and 4) can be derived from wild-type octameric AO by incubation in 80% (v/v) glycerol (lane 5, 10 μ g protein). The different AO protein bands are visualized in lane 6 (320 μ g protein of a crude extract from wild-type cells incubated in 20% glycerol in order to partially dissociate octameric AO). 40 μ g protein was loaded per lane unless otherwise indicated. O, AO octamers; S_1 , FAD-lacking subunits; S_2 , FAD-containing subunits.

Western blotting using antibodies against AO (Evers *et al.*, 1995). This way, minor amounts of monomeric AO were detected in crude extracts prepared from wild-type cells (Figure 3, lane 1, S_1); similar results were obtained after sucrose density centrifugation of crude cell extracts (Figure 2). An AO band with the same electrophoretic mobility (S_1) was also evident on Western blots of native gels prepared from both mutants containing the *rif* mutation (Figure 3, lanes 3, 4). In the Δ *per1* strain, however, a protein band with a slightly lower electrophoretic mobility (Figure 3, lane 2, S_2) was observed. The electrophoretic properties of the S_1 band were identical to those of AO monomers, obtained *in vitro* by the glycerol-mediated dissociation of octameric AO, from which FAD was chemically released (Figure 4, lane 6; Evers *et al.*, 1995). On the other hand, the properties of the S_2 AO subunits, detected in crude extracts prepared from Δ *per1* cells, were identical to the FAD-containing AO monomers obtained after *in vitro* dissociation of native AO protein (Figure 3, lane 5; Figure 4, lane 5; Evers *et al.*, 1995). Hence, the S_1 -protein band most probably represents AO monomers lacking FAD, whereas the S_2 -band represents a FAD-containing AO monomeric fraction.

The release of FAD (by KCN treatment) from octameric AO only slightly affected the electrophoretic mobility of the protein (Figure 4, lanes 2 and 4), but did not change its oligomeric state (Bruinenberg *et al.*, 1982; van der Klei *et al.*, 1989b). These observations indicate that KCN

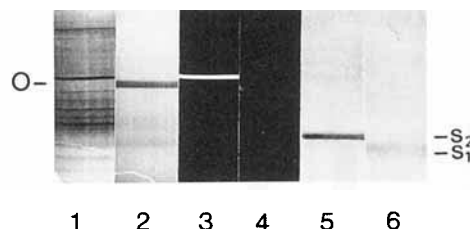


Figure 4. Coomassie staining (lanes 1–2), UV fluorescence of FAD (lanes 3–4) and Western blots, using specific α -AO antibodies after non-denaturing gel electrophoresis of crude extracts of wild-type cells before (lanes 1, 3 and 5) and after incubation with 6 mM KCN (lanes 2, 4 and 6). Incubation of wild-type AO (lane 1) with KCN results in the dissociation of FAD from the octamers; however, the protein remains octameric (lanes 2, 4) and shows approximately the same electrophoretic mobility as native AO containing FAD. Incubation of crude extracts with 80% glycerol generates a monomeric form of AO, designated S_1 (lane 5); incubation of KCN-treated crude extracts with 80% glycerol generates a band of monomeric AO, S_2 , which has a lower electrophoretic mobility than S_1 (lane 6). O, octameric AO; S_1 , FAD-lacking monomeric AO; S_2 , FAD-containing monomeric AO.

treatment does not lead to the unfolding/dissociation of AO protein.

The presence/absence of FAD in both forms of octameric AO was confirmed by fluorescence (Figure 4, lanes 3–4).

Electron microscopy

In cells of the Δ *per1.rif1* double mutant, incubated in methanol-containing media at Rf-limiting conditions, small proteinaceous aggregates were observed in the cytosol, which were specifically labelled with antibodies against AO in immunocytochemical experiments and therefore most probably represent aggregates of monomeric AO (Figure 5A). In wild-type control cells, these aggregates were never observed, instead all AO protein invariably was present inside peroxisomes (Figure 5B). In *rif1* cells, comparable aggregates were only observed in peroxisomes (Evers *et al.*, 1994); this suggests that an aggregation event, which is peroxisome-bound in the *rif1* mutant cells, takes place in the cytosol of cells of the double mutant.

DISCUSSION

We have studied the role of FAD in the assembly of the peroxisomal flavoprotein AO in intact cells of *H. polymorpha*. In previous studies, using a flavin-auxotrophic (*rif*) mutant, an unequivocal judgement on the role of FAD could not be made. This was mainly due to the fact that, under

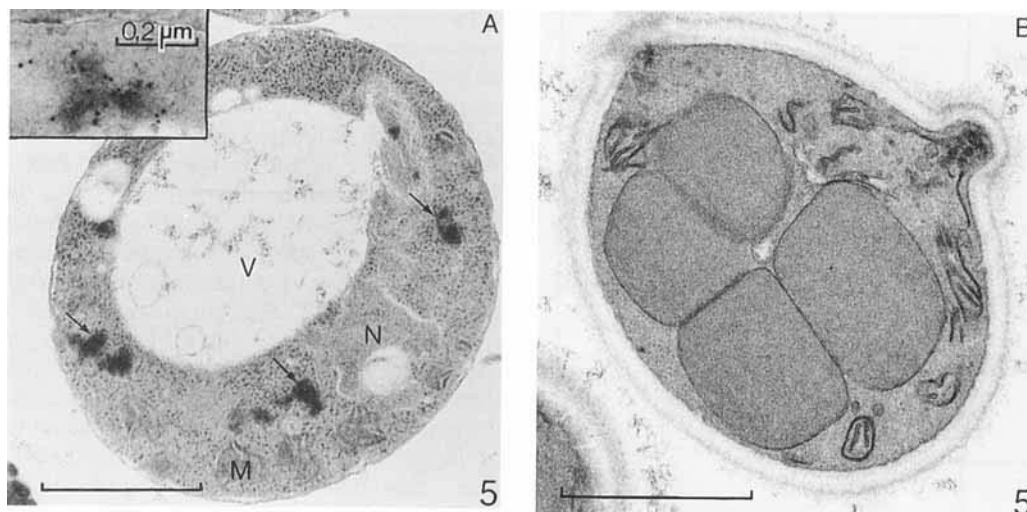


Figure 5. (A) Overall morphology of $\Delta per1.rif1$ cells, incubated in methanol-containing media. (B) Control wild-type cell containing several intact peroxisomes. In $\Delta per1.rif1$ (A) AO protein is present in the cytosol, either soluble or present in protein aggregates. Immunocytochemistry, using antibodies against AO, revealed that the gold labelling accumulated on protein aggregates (A; inset). Abbreviations: M, mitochondrion; N, nucleus; V, vacuole; AO aggregates are indicated by arrows. The bar represents 0.5 μm .

conditions of flavin limitation, a partial block of AO protein import occurred, thus leading to the spatial separation of the AO monomers (in the cytosol) and the putative AO assembly factor(s) in peroxisomes. For this reason we have now analysed the AO assembly pathway in a constructed peroxisome-deficient, Rf-auxotrophic double mutant ($\Delta per1.rif1$) in which peroxisomes were absent and consequently a peroxisomal membrane barrier was no longer present.

The overall effect of Rf-limitation on AO assembly in the *H. polymorpha* $\Delta per1.rif1$ double mutant was highly comparable to the effects observed before in the single *rif1* mutant (Evers *et al.*, 1994): accumulation of monomeric AO which lacks FAD. This form of AO protein is also present at very low levels in extracts of wild-type cells, suggesting that it forms an intermediate in AO assembly. AO monomers, lacking FAD, may easily aggregate in *H. polymorpha*. In the *rif1* mutant such aggregates were found in the peroxisomal matrix (Evers *et al.*, 1994); as evident from this study, in the $\Delta per1.rif1$ double mutant cells grown at Rf-limitation, they were occasionally formed in the cytosol. A possible explanation is that AO protein forms aggregates upon release from the putative peroxisomal FAD-binding factor in the absence of FAD (see Figure 6). As a result, AO aggregates are found inside

peroxisomes in *rif1*, whereas $\Delta per1.rif1$ these are present in the cytosol.

We propose that the binding of FAD to the AO monomers is the initial and crucial step in the *in vivo* AO assembly pathway, thus leading to the formation of an oligomerization competent FAD-containing AO subunit. In wild-type cells this step may occur inside peroxisomes, as can be deduced from the findings that FAD-containing monomers are not detectable in the cytosol of Rf-limited *rif1* cells, but on the other hand are evident in the cytosol of mutants lacking peroxisomes ($\Delta per1.rif1$ and $\Delta per1$), thus under conditions where the peroxisomal membrane barrier is no longer present.

We previously demonstrated that FAD-containing monomers are able to assemble spontaneously *in vitro* into the octameric, active conformation (Evers *et al.*, 1995). The assembly efficiency was shown to be dependent on the concentration of these monomers (Evers *et al.*, 1995). Therefore, the presence of distinct amounts of FAD-containing AO monomers in $\Delta per1$ cells is most probably due to a concentration effect, since these monomers are now diluted over the cytosol, instead of being accumulated in the peroxisomal lumen.

Our findings now also offer an explanation for the yet unexplained failure of *H. polymorpha* AO

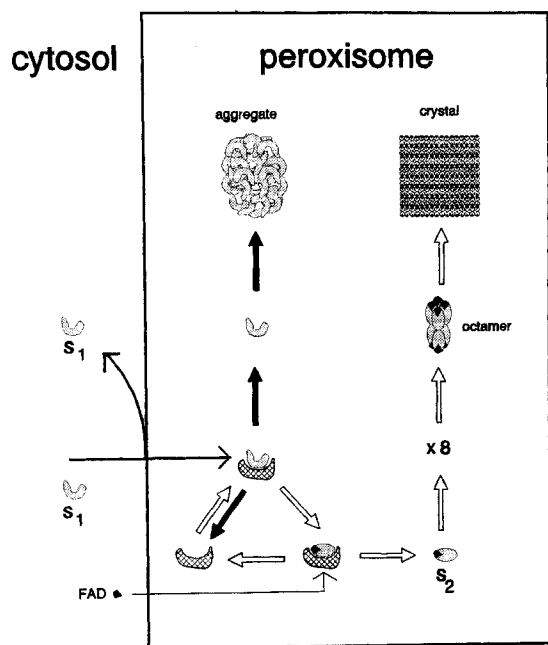


Figure 6. Hypothetical model of AO assembly and AO aggregation in wild-type and *rif1* mutant cells of *H. polymorpha*. In wild-type cells, monomeric precursors of AO (S_1) are synthesized in the cytosol and subsequently imported into peroxisomes; in the organellar matrix they interact with a yet unknown peroxisomal factor (◡) which mediates FAD-binding, thus resulting in an assembly-competent, FAD-containing monomer (S_2). After this step, oligomerization into the AO octamer and subsequent crystallization in the peroxisomal matrix may occur spontaneously. In the absence of FAD, slow dissociation of FAD-lacking AO monomers from the peroxisomal FAD-binding factor leads to partial aggregation of the protein in mutants containing the *rif1* mutation (filled black arrows) and a subsequent partial inhibition of import due to the saturation of this binding factor.

to oligomerize in peroxisomes of *Saccharomyces cerevisiae* (Distel *et al.*, 1987). As shown before, AO monomers inside bakers' yeast peroxisomes lack FAD (van der Klei *et al.*, 1989a), apparently due to the fact that the specific protein factor, essential for this, is not effective for AO. The alternative, namely that FAD is limiting in *S. cerevisiae* peroxisomes, is less likely since homologous *S. cerevisiae* flavoproteins (e.g. acyl CoA oxidase and uricase) were normally active (data not shown). The results described for *S. cerevisiae* are therefore consistent with our present findings in that, as in *H. polymorpha*, the accumulation of FAD-lacking AO monomers inside peroxisomes led to a partial block of AO import (Distel *et al.*, 1987).

In conclusion, we propose that only the FAD-containing AO subunit is able to assemble in the active octameric protein. This FAD-binding is most probably mediated by a specific protein factor which is located in the peroxisomal matrix. Whether this factor is specific for AO or also functions for other flavoproteins (e.g. D-amino acid oxidase) is yet unknown. However, a certain species specificity probably exists, since in *S. cerevisiae* peroxisomes FAD-binding to *H. polymorpha* AO had not occurred (van der Klei *et al.*, 1989a). Also, the FAD-binding factor is most probably not a typical molecular chaperone (belonging to the hsp protein families), since hsps were not detected in purified peroxisomal fractions of *H. polymorpha* (Titorenko *et al.*, 1995). Moreover, this factor does not require the specific peroxisomal environment (Nicolay *et al.*, 1987) to function, since after removal of the peroxisomal membrane barrier (i.e. in a *per*-mutant), AO assembly can also occur effectively in the cytosol (van der Klei *et al.*, 1991a). So far, we have not found evidence for the translocation of oligomeric AO into peroxisomes of *H. polymorpha*, as is observed for other proteins in peroxisomes of *S. cerevisiae* and *Yarrowia lipolytica* (McNew and Goodman, 1994; Glover *et al.*, 1994). Further studies to isolate the FAD-binding factor are in progress. A hypothetical model of the *in vivo* assembly pathway of AO in *H. polymorpha* is presented in Figure 6.

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